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Molecular signatures in response to Isoliquiritigenin in lymphoblastoid cell lines

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ABSTRACT

Isoliquiritigenin (ISL) has been known to induce cell cycle arrest and apoptosis of various cancer cells. However, genetic factors regulating ISL effects remain unclear. The aim of this study was to identify the molecular signatures involved in ISL-induced cell death of EBV-transformed lymphoblastoid cell lines (LCLs) using microarray analyses. For gene expression and microRNA (miRNA) microarray experiments, each of 12 LCL strains was independently treated with ISL or DMSO as a vehicle control for a day prior to total RNA extraction. ISL treatment inhibited cell proliferation of LCLs in a dose-dependent manner. Microarray analysis showed that ISL-treated LCLs represented gene expression changes in cell cycle and p53 signaling pathway, having a potential as regulators in LCL survival and sensitivity to ISL-induced cytotoxicity. In addition, 36 miRNAs including five miRNAs with unknown functions were differentially expressed in ISL-treated LCLs. The integrative analysis of miRNA and gene expression profiles revealed 12 putative mRNA–miRNA functional pairs. Among them, miR-1207-5p and miR-575 were negatively correlated with p53 pathway- and cell cycle-associated genes, respectively. In conclusion, our study suggests that miRNAs play an important role in ISL-induced cytotoxicity in LCLs by targeting signaling pathways including p53 pathway and cell cycle.

1. Introduction

Isoliquiritigenin (ISL) is a flavonoid that is found in licorice, shallot, and bean sprouts [1]. It has been known that ISL prevents inflammation [2], platelet aggregation [3], and tumor development [4]. In particular, many studies have shown that ISL treatment induces a cell cycle arrest or programmed cell death (apoptosis) in various cancer cells, including hepatocellular carcinoma [5], lung cancer [6,7], cervical carcinoma [8], and prostate cancer [9,10], indicating its potential as a cancer therapeutic agent. The anti-cancer potential of ISL has been reported to be accompanied through a cell cycle arrest or apoptosis regulated by various genes, such as Fas ligand (FasL)/Fas [5,6,10], Bax [5,8,10], Noxa [5], p53 [5,6,8], p21 [5–8,11], cyclin A and B, cdc2, and cdc25C [8]. However, regulatory mechanisms of ISL effect remains to be elucidated.

Epstein–Barr virus (EBV), a member of the herpes family, is associated with the development of various cancers, such as nasopharyngeal carcinoma (NPC), Burkitt's lymphoma (BL), Hodgkin's disease (HD), and post-transplant lymphoproliferative disease (PTLD) [12]. EBV also has an ability to establish lymphoblastoid cell lines (LCLs), which are widely used for pharmacogenomic discovery, via its transformation in human B lymphocytes [13]. LCLs are suitable for studies on various phenotypes related to cancer therapeutic agent-induced cytotoxcity [14–16]. For instance, the cytotoxicity of 5-fluorouracil (5-FU), an anti-cancer chemotherapy drug, was measured with 427 LCLs from the Centre d'Etude du Polymorphisme Humain (CEPH) pedigrees and SNPs in association with 5-FU cytotoxicity were identified using the genotype data obtained from the HapMap website [16]. An LCL collection from twins and siblings has been used for treatment with different drugs such as paclitaxel, cisplatin, carboplatin, and ara-C, to determine whether a drug-induced apoptosis can be utilized as a phenotype for pharmacogenomic approach [15]. Recent studies have demonstrated that LCLs can also be a useful cell model for clinical translational research [17]. For example, it was reported that the expression level of FKBP5 gene was associated with sensitivity to ara-C in LCLs [18]. Subsequently, SNPs in a FKBP5 gene were observed in samples from ara-C treated acute myeloid leukemia patients [19].

In this study, we determined the molecular signatures (genes and miRNAs) in response to ISL using LCLs, through a microarray approach. Furthermore, genes associated with the sensitivity to ISL were identified.

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2. Materials and methods

2.1. Cell culture

LCL strains were obtained from the National Biobank of Korea and cultured in an RPMI1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) and incubated at 37 °C in 5% CO_2 in air.

2.2. Cytotoxicity assay

Each of LCL strains was plated at a density of 1×10^4 cells/well in 96 well plates and various concentrations (0, 20, 40, 50, 60, 100, 120, or 140 μM) of ISL were added. After incubation for one or two days, the MTT assay was performed using Cell Titer 96® Aqueous One Solution Cell Proliferation Assay (Promega, Charbonnier, France) in accordance to the manufacturer's manual.

2.3. Total RNA extraction

For gene expression and miRNA microarray experiments, each of 12 LCL strains was seeded at a density of 5×10^6 cells on 100 mm dishes and independently treated with ISL (the concentration of 50 μM DMSO as a vehicle control for a day prior to total RNA extraction. After one day, the cell number of each LCL was measured using a trypan blue exclusion assay. Total RNA was extracted from these cells using Trizol (Invitrogen Life Technologies, Carlsbad, USA) and purified using RNeasy columns (Qiagen, Valencia, USA) according to the manufacturers' instructions.

2.4. Gene expression microarray

For gene expression profiling, the labeled cDNA, synthesized from total RNA, were hybridized to Human HT-12 expression v.4 bead array for 16–18 h at 58 °C, according to the manufacturer's instructions (Illumina, San Diego, USA). The array was scanned with an Illumina bead array Reader confocal scanner and analysis of the array image was performed through Illumina Bead Studio v3.1.3 (Gene Expression Module v3.3.8). Signal value of each gene was converted into logarithm and normalized by quantile method. After a normalization process, we obtained the signal intensity of 17, 519 (37%) of all probes (n = 47,231) on array. The comparative analysis between 12 LCL strains with and without ISL was implemented using a fold-change. A paired t-test analysis was adjusted for the false discovery rate (FDR) p-value and FDR was controlled by adjusting the p-value using a Benjamini–Hochberg algorithm. Genes differentially expressed by ISL were selected by fold-changes $\geqslant 2$ and p-value < 0.05.

2.5. The functional analysis of ISL-induced genes

The gene ontology (GO) and pathway analysis were performed with genes differentially expressed in ISL-treated LCLs compared to non-treatment LCLs, using GO database http://www.geneontology.org and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway database http://www.genome.jp/kegg respectively. The GO annotation was performed using biological processes. The significance was established at the FDR < 0.30 and p-value < 0.05.

2.6. miRNA microarray

Total RNA was labeled and hybridized to Human miRNA Microarray Release 14.0 (Agilent Technologies, Santa Clara, CA) using Human microRNA Microarray Kit in accordance to the manufacturer's manual. The array signal was scanned on an Agilent C scanner and its image was quantified using an Agilent Feature Extraction Software. Signal values of miRNAs were analyzed in the same method as the cDNA microarray. In miRNA microarray results, we obtained the signal intensities of 150 (39%) of miRNAs (n = 387) on an array via a normalization procedure (Fig. 2B).

2.7. Real-time RT-PCR

Expression of nine genes (CDC2, CD82, MCM3, MCM7, GDF15, HMOX1, NQO1, OKL38, and SLCO2B1) and four miRNAs (miR-93, miR-148a, miR-30e*, and miR-7) were quantified using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, USA) in accordance to the manufacturer's protocol. The GAPDH expression level of each sample was also quantified as an internal control. All PCR reactions were performed in duplicate experiments.

2.8. Identification of putative target genes of ISL-induced miRNAs

Target genes of ISL-induced miRNAs were predicted using two miRNA target prediction algorithms, including miRDB (http://mirdb.org) and miRBase (http://www.mirbase.org). Subsequently, putative functional miRNA–mRNA pairs with significant inverse relationship were found through the integrative analysis of miRNA and gene expression profiles. The statistical associations were evaluated between the expressions of miRNAs and their putative target genes through linear regression analysis using SPSS, version 12.0 (SPSS, Chicago, IL). The p-value of significant correlation was set at 0.05.

3. Results

3.1. Cytotoxic effect of ISL on LCLs

To determine the optimal concentration of ISL for its effect in LCLs, we first performed dose-effect experiments in 3 LCLs. As shown in Fig. 1A, the cell viability was considerably decreased in a dose-dependent manner when 3 LCLs were treated with different concentration of ISL for 2 days. The cell viability maximally declined up to 4.8% (± 1.2) at 140 μM of ISL, compared with the control group (LCLs with non-treatment). The individual variation of ISL-induced cytotoxicity is the highest in approximately 50 μM of ISL. When 12 LCLs were treated with and without 50 μM of ISL for 1 and 2 days, ISL treatment decreased the cell viability up to 55.3% (± 14.8) and 39.1% (± 9.4), respectively (Fig. 1B). For these reasons, we determined the treatment of 50 μM ISL for 1 day in LCLs for a further gene expression study.

3.2. Identification of genes differentially expressed by ISL

To identify ISL-induced genes, gene expression microarray experiments were performed using 12 LCLs with and without treatment of 50 μM ISL for 1 day. The finding revealed that the signal intensity of 414 probes had significantly decreased or increased over 2-fold changes in the ISL-treated 12 LCLs compared to their non-treatment samples (Fig. 2A). These probes included 181 down-regulated genes and 186 up-regulated genes (Fig. 2C). In particular, the expression of HMOX1 (40-fold change) and SLCO2B1 (17-fold change) increased over 10-fold in the ISL-treated LCLs (Table 1).

We carried out a GO term annotation and KEGG pathway analysis for 367 differentially expressed genes (DEGs). Thirty-nine GO terms with significant p-value (< 0.05) and FDR (< 0.3) were classified in 123 genes of all DEGs and most of the GO terms were involved in a cell cycle, indicating that cell cycle regulators play a central role in the ISL-induced cytotoxicity of LCLs (Supplementary

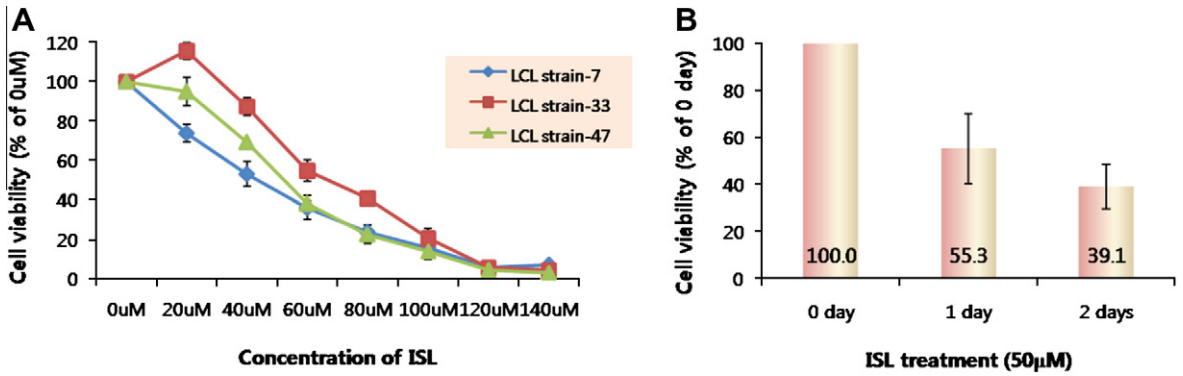

Fig. 1. The inhibitory effect of ISL on cell proliferation of LCLs. (A) Concentration-dependent effects of ISL when 3 LCL strains were treated with various concentrations of ISL for 2 days. (B) Time-dependent effects of ISL when 12 LCL strains were treated with 50 μM of ISL for 1 and 2 days.

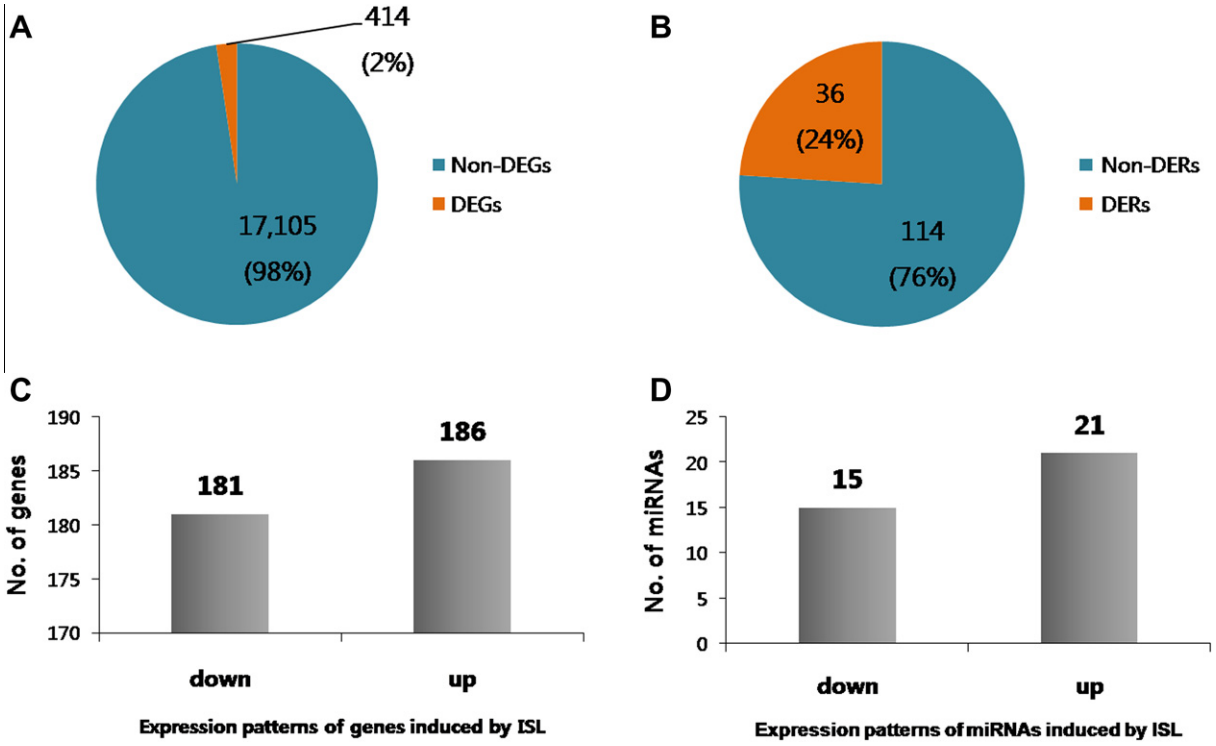

Fig. 2. Identification of differentially expressed genes (DEGs) and miRNAs (DERs) in ISL-treated LCLs. Twelve LCL strains were treated with 50 μM of ISL or vehicle alone for a day. These LCL samples were used for gene expression and miRNA microarray experiments. (A) From gene expression microarray, normalized signal intensities of 17,519 mRNA transcripts were obtained. Among them, 414 (2%) mRNA transcripts were differentially expressed over 2-fold in ISL-treated LCLs, compared to LCLs with non-treatment. (C) There was 367 individual genes. (B) Normalized miRNA microarray data contained the expression levels of 150 miRNAs. (B, D) Among them, 36 miRNAs were down- or up-regulated by ISL.

Table 1). KEGG pathway analysis showed that 31 genes were significantly included in three pathways: cell cycle, p53 signaling pathway, and oocyte meiosis (Table 2).

To validate microarray data, nine genes of the DEGs were selected for real-time RT-PCR experiments: including the top five genes (HMOX1, SLCO2B1, NQO1, OKL38, and GDF15) and four signal pathway (p53 pathway and the cell cycle)-associated genes (MCM3, CDC2, CD82, and MCM7). As shown in Fig. 3, all genes exhibited similar expression patterns with microarray results. In particular, HMOX1, SLCO2B1, and OKL38 expressions increased over 50-fold in ISL-treated LCLs.

3.3. Identification of miRNAs differentially expressed by ISL

ISL-induced miRNAs were discovered in 12 LCLs using miRNA microarray approach. The results showed that 36 miRNAs were decreased or increased over 2-fold in ISL-treated 12 LCLs compared to corresponding non-treatment samples; 15 miRNAs were decreased and 21 miRNAs were increased (Fig. 2D, Table 3). They included five miRNAs (such as miR-1268, miR-1973, miR-1249, miR-940, and miR-1977) whose functions were not known yet. Expression of 3 miRNA clusters, including miR-106b∼25, miR-106a∼363, and miR-17∼92, showed a tendency to decrease when ISL was treated in LCLs (Supplementary Table 2), indicating these miRNA clusters influence ISL-induced cytotoxicity of LCLs. In gene expression microarray data, the expression of MCM7, host gene of miR-106b∼25, was decreased (fold change = −2.3) and UNKL, putative target gene of miR-93, was increased (fold change = 2.1) in ISL-treated LCLs. These results suggest that the miR-106b ∼ 25 cluster cooperates with the MCM7 expression and miR-93 induces the down-regulation of UNKL in response to ISL.

miRNA microarray data was validated through a real-time PCR experiment for 4 miRNAs, including miR-93, miR-148a, miR-30e*, and miR-7 which randomly selected in all differentially expressed

Table 1
Top 20 genes with the highest expression changes in 12 ISL-treated LCLs, compared to non-treatment LCLs.

Gene symbol	Accession no.	Fold change
HMOX1	NM_002133	40.2
SLCO2B1	NM_007256	16.5
NQO1	NM_000903	9.6
LOC100129781	XM_001717065	8.3
OKL38	NM_013370	8.0
GDF15	NM_004864	7.8
PANX2	NM_052839	7.2
–	XM_373666	6.8
LOC729009	XR_042330	6.6
RN7SK	NR_001445	6.4
FTHL8	NR_002203	6.2
MT1G	NM_005950	6.1
C17orf91	NM_001001870	6.0
SRXN1	NM_080725	6.0
LOC392437	XR_037197	6.0
FTHL3	NR_002201	5.6
FTHL12	NR_002205	5.4
FTHL11	NR_002204	5.4
RRAD	NM_004165	5.3
MAL	NM_022440	5.3

Table 2
KEGG pathway analysis for genes differentially expressed by ISL (p-value < 0.05, FDR < 0.30).

KEGG pathway	No. of Genes	Gene symbol	p-Value	FDR
Cell cycle	23	Up-regulated genes: CDKN1A (p21), GADD45A Down-regulated genes: CDC45L, CHEK1, E2F2, MAD2L1, TTK, BUB1, BUB1B, BUB3, CDC2 (CDKN1), CDC20, CDC25C, CCNA2 (Cyclin A2), CCNB1 (Cyclin B1), CCNB2 (Cyclin B2), ESPL1, MCM2, MCM3, MCM4, MCM6, MCM7, PLK1	<0.001	<0.001
p53 signaling pathway	12	Up-regulated genes: CD82, CDKN1A (p21), CYCS, GADD45A, PMAIP1 (NOXA), SESN1 Down-regulated genes: CHEK1, GTSE1, CDC2 (CDKN1), CCNB1 (Cyclin B1), CCNB2 (Cyclin B2), RRM2	<0.001	<0.001
Oocyte meiosis	11	Down-regulated genes: FBXO5, MAD2L1, AURKA, BUB1, CDC2 (CDKN1), CDC20, CDC25C, CCNB1 (Cyclin B1), CCNB2 (Cyclin B2), ESPL1, PLK1	<0.001	0.099

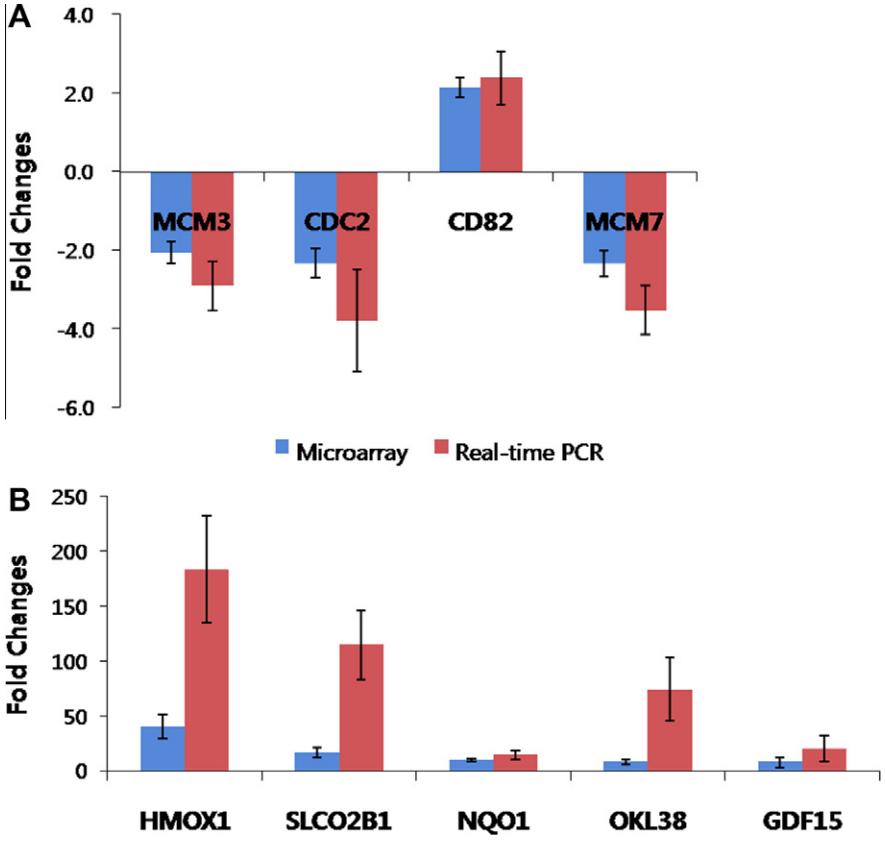

Fig. 3. Validation of gene expression microarray data. Real-time PCR experiments were performed for 9 genes including (A) signal pathway (p53 pathway and cell cycle)-associated genes (MCM3, CDC2, CD82, and MCM7) and (B) top 5 genes (HMOX1, SLCO2B1, NQO1, OKL38, and GDF15) with the highest expression changes.

miRNAs (Fig. 4). The expression of these miRNAs decreased in ISL-treated LCLs, similar to the microarray data.

3.4. Integrative analysis of differentially expressed genes and miRNAs

To elucidate miRNA-related transcriptional regulatory machinery in ISL-treated LCLs, predictive miRNA–mRNA functional pairs were identified using differentially expressed genes (n = 367) and miRNAs (n = 36). Target genes of each of 36 miRNAs with different expressions were predicted using two online databases (miRBase and miDB) and miRNA–mRNA pairs with inverse correlation were found.

As shown in Table 4, 12 miRNA–mRNA pairs (11 miRNAs and 12 mRNAs) showed a significant negative correlation (p-value < 0.05).

Table 3
miRNAs differentially expressed by ISL.

Up-regulated	Fold change	Down-regulated	Fold change
hsa-miR-630	14.4	hsa-miR-33a	−2.0
hsa-miR-188-5p	13.8	hsa-miR-19b-1*	−2.1
hsa-miR-638	12.8	hsa-miR-25	−2.1
hsa-miR-1207-5p	11.6	hsa-miR-130b	−2.2
hsa-miR-1246	11.4	hsa-miR-93	−2.2
hsa-miR-1225-5p	9.6	hsa-miR-148a	−2.3
hsa-miR-1915	9.0	hsa-miR-17*	−2.4
hsa-miR-1202	8.8	hsa-miR-18b	−2.5
hsa-miR-1275	6.3	hsa-miR-223	−2.5
hsa-miR-762	6.0	hsa-miR-155*	−2.6
hsa-miR-1268	5.1	hsa-miR-30e*	−2.6
hsa-miR-494	4.8	hsa-miR-21*	−2.7
hsa-miR-320c	4.3	hsa-miR-7	−2.8
hsa-miR-1973	4.3	hsa-miR-18a	−3.2
hsa-miR-1290	3.9	hsa-miR-886-3p	−3.3
hsa-miR-483-5p	3.4		
hsa-miR-1249	3.2		
hsa-miR-760	2.8		
hsa-miR-575	2.6		
hsa-miR-940	2.4		
hsa-miR-1977	2.2		

Note): Bold letters indicate miRNAs with unknown function.

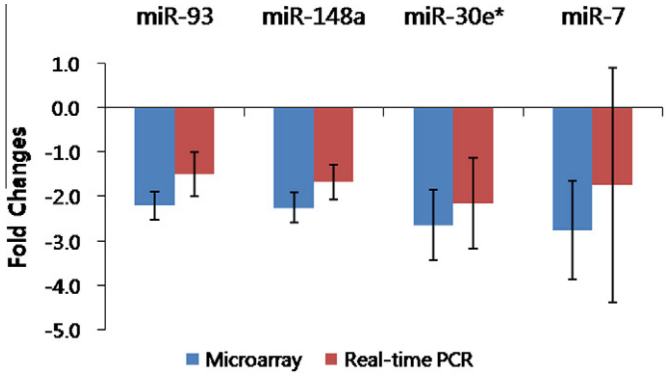

Fig. 4. Real-time PCR analysis for validation of miRNA microarray data.

Table 4
Putative miRNA–mRNA functional pairs differentially expressed by ISL (p-value < 0.05).

miRNA expression		mRNA expression		miRNA-mRNA pair	
miRNA	Fold change	mRNA	Fold change	R	p-value
miR-630	14.4	BTLA	−2.0	−0.71	0.009
miR-1207-5p	11.6	CDK5R1	−2.8	−0.62	0.033
miR-1202	8.8	C20orf103	−2.6	0.79	0.002
miR-575	2.6	NCAPG	−2.8	−0.70	0.012
miR-575	2.6	CDC45L	−2.3	−0.60	0.038
miR-7	−2.8	TPP1	2.5	−0.61	0.035
miR-21*	−2.7	CLIP4	2.5	−0.59	0.044
miR-30e*	−2.6	RARRES3	2.1	−0.77	0.003
miR-148a	−2.3	UNKL	2.1	0.58	0.047
miR-93	−2.2	UNKL	2.1	0.62	0.032
miR-130b	−2.2	ZDHHC14	2.1	0.58	0.048
miR-25	−2.1	ANKRD24	2.5	0.69	0.014

For example, CDK5R1 [20], a p53 signaling pathway associated gene, was paired to be a putative target of miR-1207-5p while CDC45L [21–23], a cell cycle associated gene, to be a predictive target of miR-575. These results indicate that miR-1207-5p and miR-575 may control p53 pathway and the cell cycle in LCLs through the expression regulation of their target genes, respectively.

4. Discussion

To identify molecular signatures associated with ISL-induced cell death, gene expression and miRNA microarray experiments were performed with ISL-treated 12 LCLs and corresponding non-treated samples. This study showed that 367 genes and 36 miRNAs were differentially expressed in response to ISL treatment (Fig. 2), suggesting that these differential transcripts may be responsible for an inhibitory effect of ISL on cell proliferation of LCLs.

It has previously been reported that ISL induced cell cycle arrest or cell death via p53 pathway in various cancer cells such as hepatoma HepG2 cells [5], lung cancer A549 cells [6], cervical carcinoma HeLa cells [8], and uterine leiomyoma cells [11]. Our study confirmed that ISL induce cell death of LCLs in a dose and time-dependent manner (Fig. 1). Gene expression microarray data exhibited that many p53 pathway associated genes (e.g., CD82, p21, GADD45A) and cell cycle associated genes (e.g., p21, GADD45A, CDC45L, CDC2, PLK1) were involved in the ISL-induced cell death of LCLs (Table 2). Taken together, these findings suggest that ISL may be used as an effective therapeutic agent for various proliferative diseases by targeting the p53 pathway or cell cycle.

Expressions of growth differentiation factor 15 (GDF15) [24–29] and heme oxygenase 1 (HMOX1) [30] are increased when cells undergo apoptosis medicated by various anti-cancer and apoptosis-inducing agents. In this study, we found that HMOX1, SLCO2B1, and OKL38 were increased over 50-fold in response to the ISL treatment in LCLs, determined by real-time RT-PCR experiments. These results suggest that HMOX1, SLCO2B1, and OKL38 have a possibility as target genes for cancer therapy. In addition, HMOX1 has anti-inflammatory activity [31,32] and was increased in ISL-treated RAW 264.7 macrophages [32], suggesting that this gene may play an important role in the anti-inflammatory effect of ISL.

There were no reports of miRNAs associated with ISL effects. In this study, we found 36 miRNAs differentially expressed in response to ISL in LCLs. These miRNAs may play an important role in the cell survival and cytotoxicity of LCLs in response to ISL treatment. Furthermore, our findings provide the first functional information of five miRNAs containing miR-1268 (5-fold), miR-1973 (4-fold), miR-1249 (3-fold), miR-940 (2-fold), and miR-1977 (2-fold).

miRNAs have been known to regulate the expression of their target genes negatively. Here, we have identified 12 putative miRNA–mRNA functional pairs with inverse relationships using pair-wise correlation analyses of gene expression and miRNA microarray data. CDK5R1 and CDC45L were down-regulated in ISL-treated LCLs, compared with non-treated LCLs (Table 4). CDK5R1 encodes p35, which activates cyclin-dependent kinase 5 (Cdk5), a mediator of p53 stabilization and activation [20]. CDC45L encodes cell division cycle protein 45 (CDC45), which play an essential role in DNA replication [21–23]. Furthermore, CDK5R1 and CDC45L showed an inverse correlation with miR-1207-5p and miR-575, respectively (Table 4). These results suggest that miR-1207-5p and miR-575 may be involved in ISL-induced cytotoxicity of LCLs through the negative regulation of their target genes related to the p53 pathway and cell cycle.

In this study, we first demonstrated ISL inhibitory effect on cell proliferation in LCLs. Microarray data showed putative candidate genes (n = 367) and miRNAs (n = 36) to regulate ISL effect, having a potential as regulators in LCL survival and sensitivity to ISL-induced cytotoxicity. Furthermore, we found 12 putative miRNA-mRNA functional pairs with significant negative correlation, contributing to understanding the molecular mechanisms of

inhibitory effect of ISL on cell proliferation as well as miRNA-medicated transcriptional regulatory mechanisms. miR-1207-5p and miR-575 revealed a potential as regulators of p53 pathway and cell cycle in LCLs, respectively.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.09.070.

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